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Box–Behenken-Supported Development and Validation of UPLC Method for the Estimation of Eugenol in *Syzygium aromaticum, Cinnamomum tamala,* and *Myristica fragrance*

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Abstract: Eugenol (EUG) is one of the most important components available in several spices, including clove, bay leaves, and nutmeg. These spices are used as flavouring agents in foods and beverages. The aim of the present study is to develop and validate a rapid, simple, sensitive, and robust ultra-performance liquid chromatography (UPLC) technique for the quantitative estimation of EUG in the ultrasound-assisted methanolic extracts of three spices, namely *Syzygium aromaticum* (L.) Merr. & L.M.Perry (SA), *Cinnamomum tamala* (Buch.-Ham.) T.Nees & Eberm (CT), and *Myristica fragrance* Houtt. (MF). EUG was isocratically separated on a UPLC C_{18} column. The acetonitrile:methanol:water (50:40:10, *v*/*v*/*v*) solvent in different proportions was optimized as the mobile phase for the determination of EUG in ultrasound-assisted methanolic extracts of three different spices. The quantitative estimation of EUG was performed at a 281 nm detection wavelength. The column oven temperature was maintained at 35 ± 5 °C, and the flow rate of the mobile phase was 0.2 mL/min using an injection volume of 1 µL. The UPLC technique was validated according to the ICH guidelines and showed an excellent linearity range of 10–100 ng/mL. The robustness of the method was validated using Box–Behenken response surface design (BBD) software, and a 0.2 mL/min flow rate of the mobile phase, a column oven temperature of 308 K, and a 281 nm detection wavelength were found to be the best optimal conditions for obtaining the highest amount and separation of EUG. The content of EUG in ultrasound-assisted methanolic extracts of SA, CT, and MF using the UPLC technique showed 313.67 ± 0.87 mg g^{-1}, 44.95 ± 0.56 mg g^{-1}, and 59.66 ± 0.41 mg g^{-1}, respectively. The antioxidant potentials of EUG, SA, CT, and MF were analysed using the DPPH (2,2-diphenyl-1-pcrylhydrazil radical) method, which revealed the antioxidant potential of EUG (IC_{50} = 3.12 µg/mL), standard ascorbic acid (IC_{50} = 7.06 µg/mL), SA ultrasound-assisted methanolic extract (IC_{50} = 5.97 µg/mL), CT ultrasound-assisted methanolic extract (IC_{50} = 49.48 µg/mL), and MF ultrasound-assisted methanolic extract (IC_{50} = 65.16 µg/mL). The proposed UPLC technique can be used to quantitatively assess various spices, plants, pharmaceutical products, and polyherbal formulations containing EUG as an active constituent.

Keywords: clove; spices; antioxidant; UPLC; DPPH

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1. Introduction

Eugenol (EUG, 4-allyl-2-methoxyphenol) (Figure 1) is a natural phenolic compound present in essential oils, especially in clove oil. EUG is used as a spice and flavouring agent because of its strong aroma. EUG is also used as a dental antiseptic [1,2]. EUG has been widely utilised as a medicinal remedy in the treatment of dental pain, odontitis, and hyperalgesia because of its local anaesthetic properties [3,4]. EUG exhibits numerous pharmacological properties, including antioxidant, anti-inflammatory, anticancer [5,6], anticonvulsant [7], and antibacterial and antifungal properties [8,9]. EUG-induced ROS reduces neuromuscular transmission [10] and CNS function [7]. EUG exhibited cardio-protection against ischemic injury [11] and also produced hypotension and bradycardia

after IV administration in rats [12]. It is beneficial in the treatment of isoproterenol-induced cardiac hypertrophy [13]. EUG is lipophilic in nature and crosses the blood–brain barrier. EUG protects neuronal cells against NMDA-induced excitotoxicity [14]. EUG also exhibited antipyretic [15], antinociceptive [16], antistress and antidepressant activity [14]. EUG is frequently used as a fish anaesthetic. A marketed formulation consisting EUG as a chief constituent has been reported as a safe, cost-effective, and very effective fish anaesthetic [17]. EUG has been used for fish handling and shipping in Australia and European countries and is also recommended for fish euthanasia in Brazil [18,19]. The Food and Agricultural Organization and WHO recommend a daily dose of 2.5 mg/kg body weight of EUG [20,21].

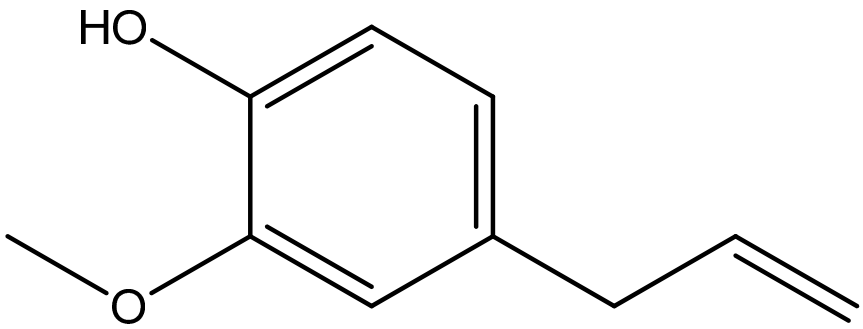

Figure 1. Chemical structure of EUG.

Syzygium aromaticum (L.) Merr. & L.M.Perry (SA), popularly known as clove, is a most valuable condiment, belongs to the family Myrtaceae, and has been used as a food preservative and medicine since ancient times. SA is most extensively used in Indian and Chinese traditional medicine as a stimulating agent. It has also been used since ancient times for the treatment of nausea, vomiting, liver disease, gastrointestinal tract ailments, scabies, cholera, malaria, TB, and many bacterial and protozoan infections [22]. The essential oil of SA is used to treat burns, wounds and toothaches and is used in perfumes and soaps. SA oil is most widely used as a natural anaesthetic due to its efficacy at a small dose, nonspecific toxicity, and easy availability, all of which are advantages of SA oil application in aquaculture [23]. SA oil is utilized as a natural anaesthetic agent for fishery management research [24]. SA contains quercetin, kaempferol, caffeic, ferulic, gallic, and ellagic acid, eugenol, isoeugenol, and tannins [25]. Eugenol (EUG) is the main active constituent of SA and is present in the concentration range of 80–85% [26].

Cinnamomum tamala (Buch.-Ham.) T.Nees & Eberm (CT) is commonly known as tezpat or bay leaf (family Lauraceae). It is indigenous to the Himalayan region of India and is also found in Nepal, Bhutan and China. In ancient times, the plant was used in the traditional formulations of Ayurveda. It is generally used in the food and perfumery industries due to its aromatic properties [27]. It is also used in pharmaceutical preparations for the treatment of vaginitis, rheumatism, and neuralgia. Its dried leaves are used commercially for culinary preparations. It contains cinnamaldehyde, 1–8 cineole, limonene myrcene, camphene, and eugenol. [28]. CT exhibits several pharmacological properties, such as antidiabetic, anti-inflammatory, anti-diarrhoeal, anthelmintic, antifungal, anticonvulsant, and antibacterial properties. The leaves of CT are most commonly used in foods, drinks, meats, and fast foods as a flavouring agent [29]. *Myristica fragrance* Houtt. (MF) is popularly known as "Rou Dou Kou" or nutmeg; it is in the family Myristicaceae and is an evergreen aromatic tree. It is a renowned lucrative source of mace and nutmeg, which is extensively used as a spice in the culinary arena. The MF plant is indigenous to Indonesia but is also distributed in India, South Africa, and the United States of America [30]. As per traditional Chinese medicine, nutmeg is commonly used in the prevention of diarrhea and gastroenteritis [31]. The seeds of MF are traditionally used for their anti-inflammatory, antithrombotic, antitumor, antioxidant, and antibacterial properties [32,33]. Furthermore, MF is used in the treatment of anxiety, diarrhea, abdominal cramps, and rheumatism in the

Ayurvedic system of medicine [34]. Malik et al. reported that MF is used in the management of hypertension in Pakistan [35]. MF extract has also been utilized as a natural anaesthetic for ornamental fish, including goldfish [36]. Chemically, nutmeg contains myristic acid, myristicin, lignane, camphene, and eugenol. Eugenol is extensively used in dentistry for oral hygiene. EUG is a highly recommended fish anaesthetic [37].

The literature has reported several analytical procedures to estimate only EUG or a combination of EUG with other compounds. Ultraviolet (UV) spectrometry was performed to quantitatively estimate EUG in personal care products [38]. Various high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) analyses were conducted to assess EUG in various plant extracts and herbal formulations [38–44]. Several gas chromatography–mass spectrometry methods have also been reported for EUG estimation in *Eugenia caryophyllata* extracts [45,46]. EUG has been detected in clove essential oil using electrochemical and voltammetry methods [47,48]. Moreover, liquid chromatography–mass spectrometry and HPLC methods were used for concurrently analysing cuminaldehyde and EUG in Chinese herbal medicine [49].

This study established a simple, reliable, precise, sensitive, and cost-effective UPLC technique for quantitatively estimating EUG available in the ultrasound-assisted methanolic extracts of spices, such as *Syzygium aromaticum* (L.) Merr. & L.M.Perry (SA), *Cinnamomum tamala* (Buch.-Ham.) T.Nees & Eberm (CT), and *Myristica fragrance* Houtt. (MF), and determined the robustness of this method by using BBD. The International Council for Hormonization (ICH) Q2-R1 guidelines were used to validate the UPLC technique for the estimation of EUG in ultrasound-assisted methanolic extracts of SA, CT, and MF. Furthermore, the antioxidant potential of EUG and methanolic extracts of different spices were examined using the DPPH method.

2. Materials and Methods

2.1. Materials

Ascorbic acid (AA) and EUG were purchased from Sigma Aldrich. HPLC-grade solvents were procured from Chromasolve (Seelze, Germany). All spices, such as SA, CT, and MF, were purchased from the hypermarket of Rakkah, Dammam, Saudi Arabia. All three dried spices were powdered in a grinder. The powdered SA, CT, and MF were used for the ultrasound-assisted methanolic extraction of EUG.

2.2. UPLC Condition

The EUG content was determined on UPLC-PDA. EUG was eluted on an Acquity H-Class UPLC–photodiode array detector (PDA) (Waters, Milford, MA, USA), and Empower 3 software was used for the chromatographic separation and identification using an Acquity UPLC CSHTM C18 1.7 µm, 2.1 × 100 mm column. The column oven temperature was maintained at 35 ± 5 °C. The mobile phase comprised acetonitrile, methanol and water in a 50:40:10 (*v*/*v*/*v*) ratio in isocratic mode at a 0.3 mL/min flow rate, and the injection volume was 1 µL. The chromatographic method's total run time was 3.0 min.

2.3. Calibration Curve (CC) of EUG

A stock solution (SS) of EUG (1 mg/mL) was prepared in methanol. The calibration curve (CC) of EUG (10–100 ng/mL) was prepared using the three replicated plots of the concentration versus the area of the peak by employing the Empower software.

2.4. Ultrasound-Assisted Extraction of Spices

The ultrasound-assisted extraction of the dried spices (SA, CT, and MF) was performed using ultrasonic vibrations with the ultrasonic model WUC-D10H Daihan Scientific, Korea. The powders of SA, CT, and MF (1 g each) were sonicated for 1 h at 45 °C in 50 mL of methanol. The extraction procedure was repeated three times to ensure complete extraction. These extract solutions were used as the sample solutions for the quantitative assessment of EUG in ultrasound-assisted methanolic extracts of *Syzygium aromaticum* (L.) Merr. &

L.M.Perry (SA), *Cinnamomum tamala* (Buch.-Ham.) T.Nees & Eberm (CT), and *Myristica fragrance* Houtt. (MF) by using the UPLC technique. All samples were filtered through a 0.22 µm polytetrafluroethylene (PTFE) disk filter prior to use.

2.5. Method Validation

The UPLC technique was validated according to ICH guidelines [50] for EUG assessment. Specificity refers to the capability of an analytical process to detect the desired analytes in the presence of other compounds. The specificity of the UPLC technique was confirmed by matching the retention time and peak apex in ultrasound-assisted methanolic extracts of SA, CT, and MF with that of standard EUG.

The linearity of EUG was examined by devising a graph of the concentration against the peak area. Different concentrations of the EUG solution with respect to the peak area were utilised to construct the calibration curve (CC) using the least squares linear regression method.

The precision process was validated according to ICH. To study the application of the analytical procedure to low, medium, and high concentrations of EUG, inter/intra-day precision was determined in triplicates within a day and on three different days. All relative standard deviations (RSD) in the detected constituent were calculated to assess the precision of the method.

The accuracy of the UPLC technique for EUG was estimated using the recovery study. The accuracy was determined at three concentration levels (40, 80, and 100 ng/mL) for EUG, and % recovery was determined as mean ± SD in triplicates.

The robustness of the UPLC technique was examined by slightly changing the chromatographic conditions, including the flow rate, detection wavelength (nm), and column temperature (K). In this study, method robustness was assessed by the Box–Behnken design (BBD) software (Stat-Ease, Minneapolis, MN, USA) [51,52].

For EUG, the limit of detection (LOD) and limit of quantification (LOQ) of the UPLC technique were estimated using the signal-to-noise ratio by the following given equations:

$$\text{LOD} = 3.3\ \sigma/\text{S}$$

$$\text{LOQ} = 10\ \sigma/\text{S}$$

Here, σ represents the standard deviation of the response, and S represents the slope of the calibration curve.

2.6. Antioxidant Activity

The antioxidant potentials of the extracts and standard EUG were measured using (0.004% *w*/*v*) DPPH solution in methanol [53]. The ultrasound-assisted methanolic extracts of SA, CT, MF, and standard EUG were mixed with methanol to prepare a stock solution (1 mg/mL). Ascorbic acid (AA) was used as the reference standard. Freshly prepared DPPH solution was taken in test tubes. The methanolic extracts of SA, CT, and MF (1–200 µg/mL), standard EUG, and AA (0.5–50 µg/mL) were separately added to each test tube, and the volume was brought up to 3 mL. The solutions were allowed to stand for 10 min. Then, the absorbance of these solutions was measured at 515 nm by a spectrophotometer (Shimadzu UV-Vis 1601). The control sample was prepared with the same materials without the extracts and AA. Percentage inhibition was calculated using the following formula, and IC_{50} was computed by employing Graph Pad Prism version 5.03.

$$\%\ \text{Inhibition} = A_0 - A_1/A_0 \times 100$$

3. Results and Discussion

3.1. Optimization of UPLC Condition

To obtain a chromatogram with high EUG resolution in a short time duration, the column, mobile phase composition, flow rate of the mobile phase, and detection wavelength

were optimized. Different ratios of several mobile phases were analysed, and acetonitrile:methanol:water (50:40:10, $v/v/v$) was found to present the maximum separation for EUG at 1.53 min using the UPLC C_{18} column at a flow rate of 0.3 mL/min. The oven temperature was kept at 35 ± 5 °C within 3 min of the total run time for the ultrasound-assisted methanolic extracts of SA, CT, and MF (Figures 2–4). Figure 5 represents the process of matching the spectrum of EUG with the ultrasound-assisted methanolic extracts of SA, CT, and MF. A detection wavelength of 281 nm was suitable for this EUG analysis.

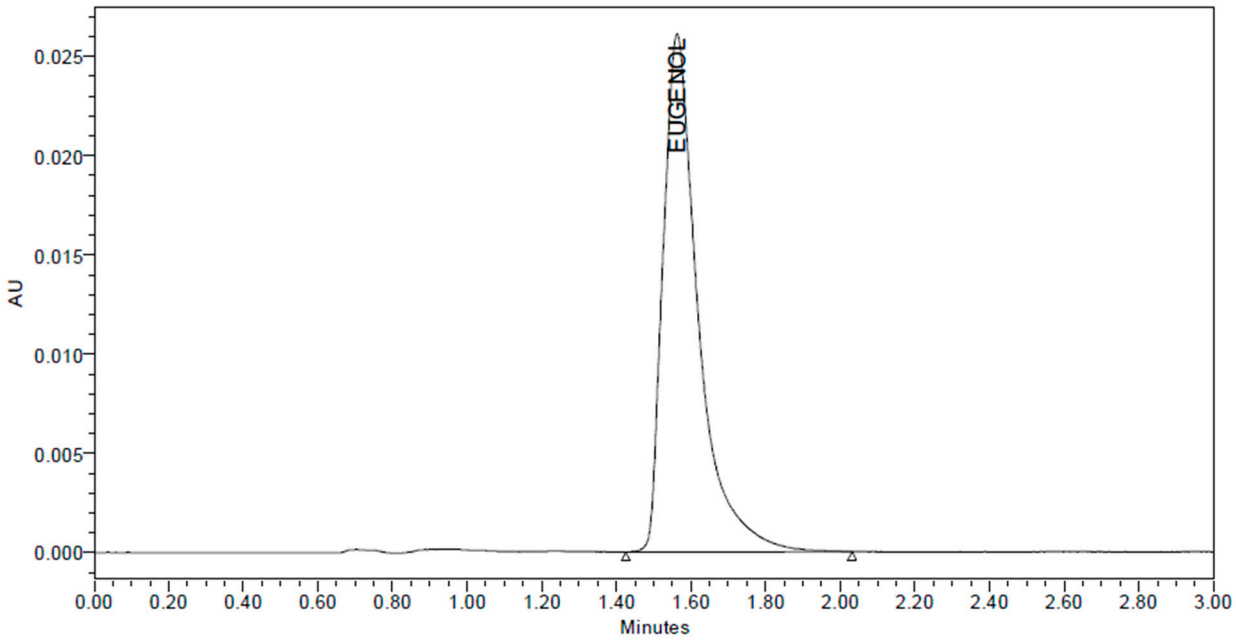

Figure 2. UPLC chromatogram of standard EUG.

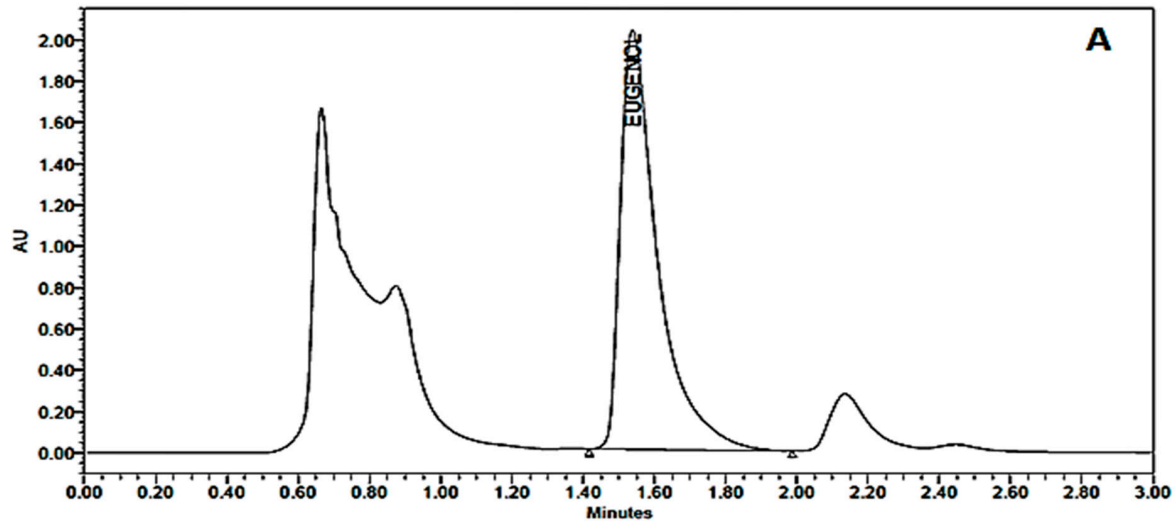

Figure 3. *Cont.*

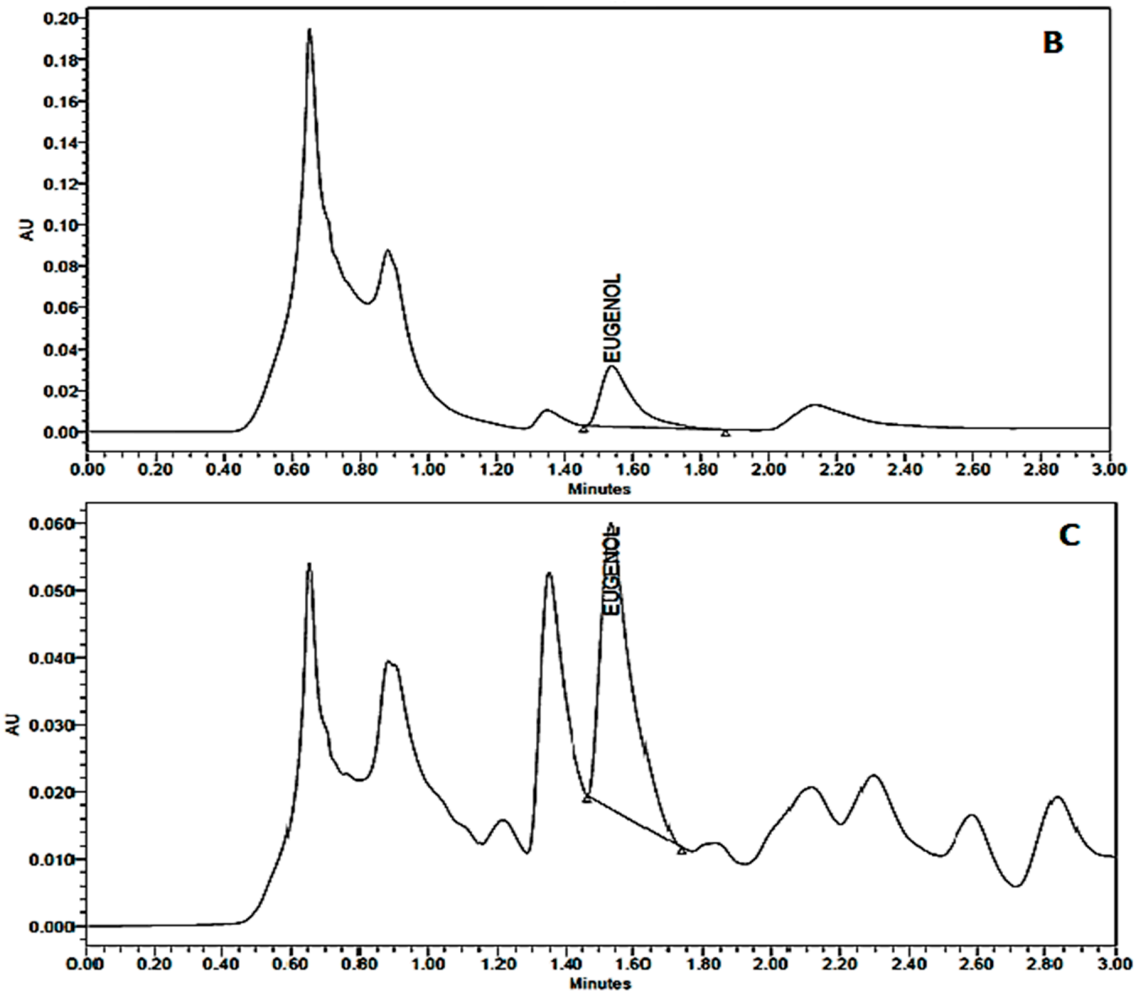

Figure 3. UPLC chromatogram of ultrasound-assisted methanolic extract of (**A**) *Syzygium aromaticum* (SA), (**B**) *Cinnamomum tamala* (CT), and (**C**) *Myristica fragrance* (MF).

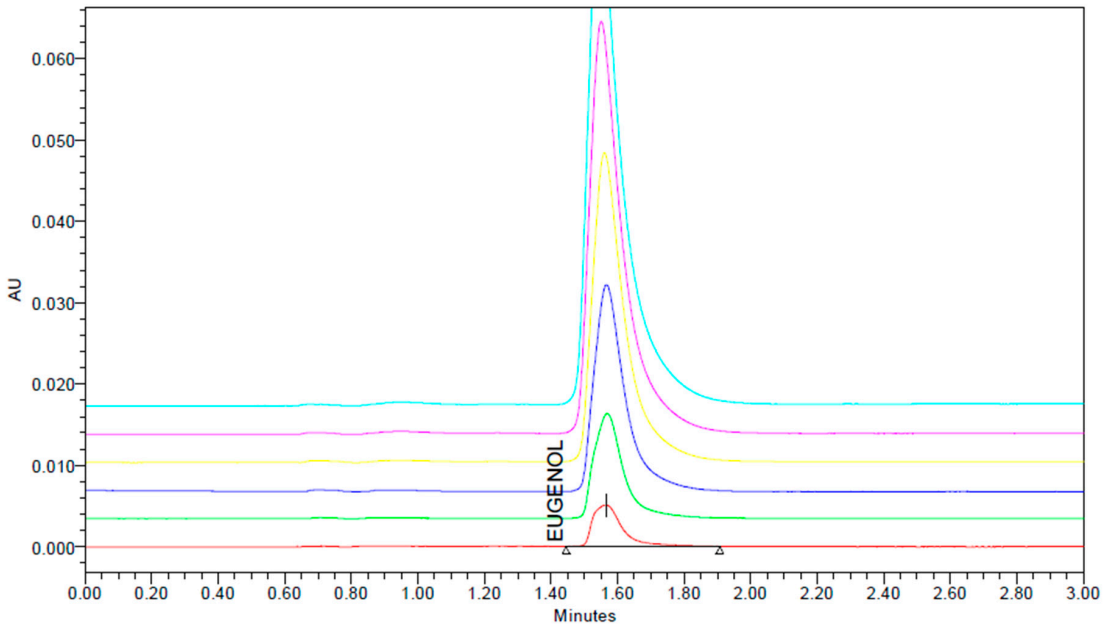

Figure 4. UPLC overlay chromatogram of EUG.

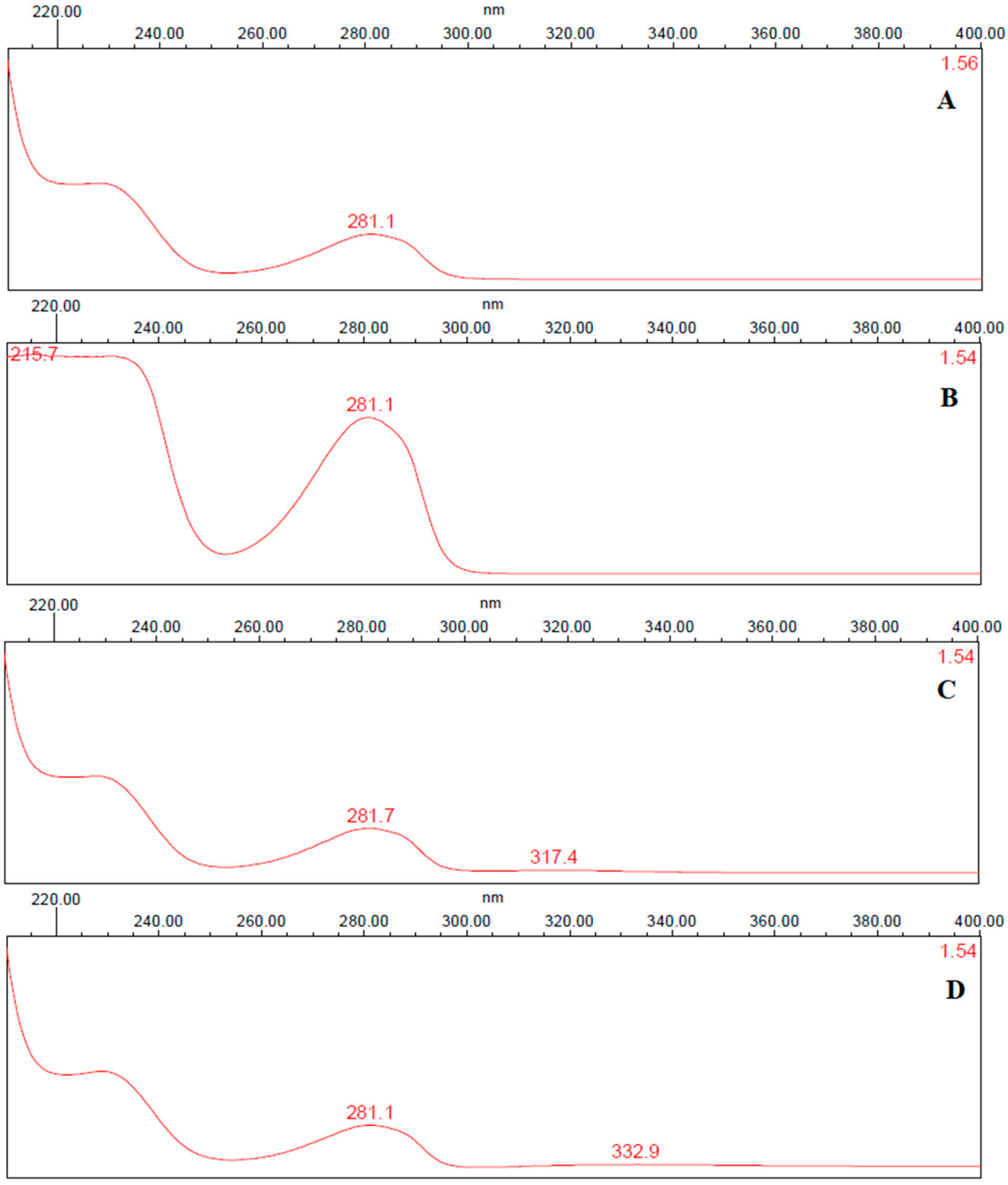

Figure 5. Spectra of eugenol. (**A**) Standard eugenol (EUG), (**B**) methanolic extract of *Syzygium aromaticum* (SA), (**C**) methanolic extract of *Cinnamomum tamala* (CT), and (**D**) methanolic extract of *Myristica fragrance* (MF).

3.2. Method Validation

3.2.1. Specificity

The specificity of the suggested UPLC technique for determining EUG could be assessed by comparing the retention time and PDA spectrum of the EUG in the ultrasound-assisted methanolic extracts of SA, CT, and MF with standard EUG. The collective PDA

spectrum of standard EUG and the EUG in the ultrasound-assisted methanolic extracts of SA, CT, and MF at 281 nm are presented in Figure 5 for comparison. The standard and the ultrasound-assisted methanolic extracts of SA, CT, and MF all had identical spectra, with a retention time at 281 nm, demonstrating the specificity of the proposed UPLC technique for the estimation of EUG.

3.2.2. Linearity

Standard EUG dilutions for linearity tests were prepared with respect to sample concentrations. The EUG standard was injected at six varying concentrations in triplicate. The calibration curve (CC) of EUG using the UPLC technique is presented in Figure 6. In relation to the peak area at 281 nm, the EUG concentration was linear for 10–100 ng/mL, and the correlation coefficient R^2 was 0.9999. The LOD and LOQ were 4.81 and 14.58 ng/mL, respectively, for EUG. The obtained results of linearity, LOD and LOQ are summarized in Table 1.

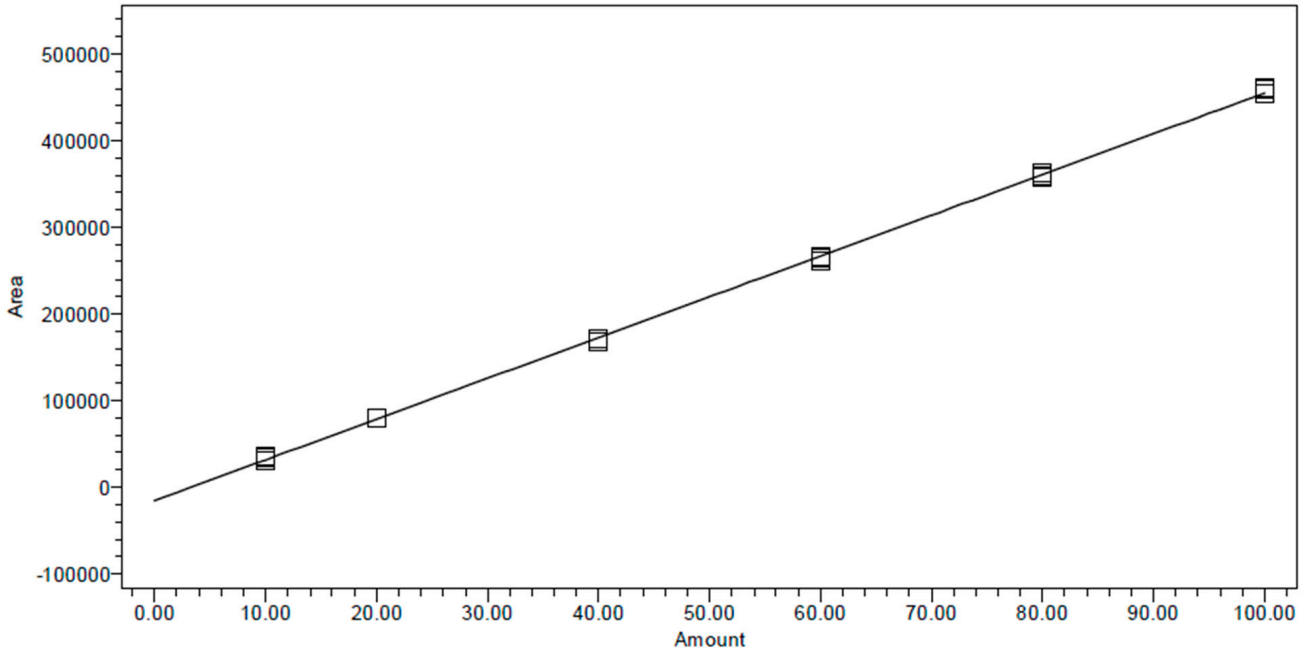

Figure 6. Calibration curve of standard EUG at different concentration levels.

Table 1. Linear regression analysis for the estimation of EUG.

Parameters	EUG
Linearity range ng/mL	10–100
Correlation coefficient (R^2)	0.9999
LOD	4.81
LOQ	14.58

3.2.3. Precision

The interday and intraday precision of the UPLC technique for EUG determination was assessed and presented as the mean area peak, S.D., and % RSD, which were found within the range according to ICH guidelines. Table 2 represents the precision results for the assessment of EUG through UPLC.

Table 2. Precision of EUG.

Amount	Inter-Day Precision		Intra-Day Precision	
ng/mL	Mean Peak Area ± SD	%RSD	Mean Peak Area ± SD	%RSD
40	171,870.4 ± 868.94	0.50	167,160.6 ± 639.60	0.38
80	362,695.2 ± 3125.71	0.86	359,949.5 ± 2927.16	0.81
100	453,913.3 ± 4432.41	0.97	464,925.2 ± 4553.41	1.00

3.2.4. Accuracy

The accuracy of the UPLC technique was assessed by conducting the recovery study with the standard addition technique. Samples with a known concentration of the EUG standard were spiked with three different concentrations. The variation among spiked and non-spiked samples was calculated and compared with the known concentration added to calculate the percentage recovery. Results are shown in Table 3. The percentage recovery of EUG estimated using the UPLC technique at three levels was 98.93–101.51%. The estimated percentage recovery of EUG was within the limit of 100% ± 2% and indicated that the UPLC technique was accurate for EUG assessment.

Table 3. Accuracy of EUG.

Excess Spike Concentration	% Recovery of EUG
40%	100.68 ± 0.39
80%	98.93 ± 0.42
100%	101.51 ± 0.55

3.2.5. Robustness

For EUG assessment, the robustness of the UPLC technique was determined by slightly modifying the flow rate, detection wavelength (nm), and column temperature (K). A three-factor BBD was performed with 17 test runs. The responses of all 17 optimized experimental runs are presented in Table 4. Stat-ease software (Design expert 13, Minneapolis, MN, USA) was used to obtain the below polynomial equation for EUG:

$$\text{Peak Area (Y)} = 1.714 \times 10^5 + 882.58\ A + 77.76\ B + 608.21\ C + 83.71\ AB + 1209.67\ AC + 140.00\ BC - 3360.42\ A^2 - 1673.90\ B^2 - 2444.66\ C^2 \quad (1)$$

Table 4. Chromatographic factors for Box–Behnken response surface design methodology.

Run	Factor 1 A: Flow Rate (mL)	Factor 2 B: Column Temperature (K)	Factor 3 C: Wavelength (nm)	Response R1 (Area)
1	0.1	308	283	163,458.925
2	0.2	313	279	165,345.223
3	0.3	308	283	167,062.413
4	0.2	313	283	168,742.113
5	0.3	303	281	167,061.771
6	0.2	308	281	171,422.988
7	0.2	308	281	171,422.988
8	0.2	303	283	168,953.194
9	0.3	308	279	165,327.116
10	0.2	303	279	166,116.307
11	0.2	308	281	171,387.46
12	0.2	308	281	171,396.543
13	0.1	303	281	164,883.025
14	0.3	313	281	168,031.302
15	0.2	308	281	171,408.863
16	0.1	313	281	165,517.714
17	0.1	308	279	166,562.316

In this EUG polynomial equation, the positive values indicate the outcome that helps optimisation. By contrast, negative values denote the contrary association between factors and responses [52,54]. This equation indicates that the factors, including the flow rate, column temperature, and wavelength, exhibit a positive effect on response (Y) (Figure 7). A mobile phase flow rate of 0.2 mL/min, a column oven temperature of 308 K, and a 281 nm detection wavelength were found to be the best optimal conditions for obtaining the highest amount and separation of EUG as per BBD.

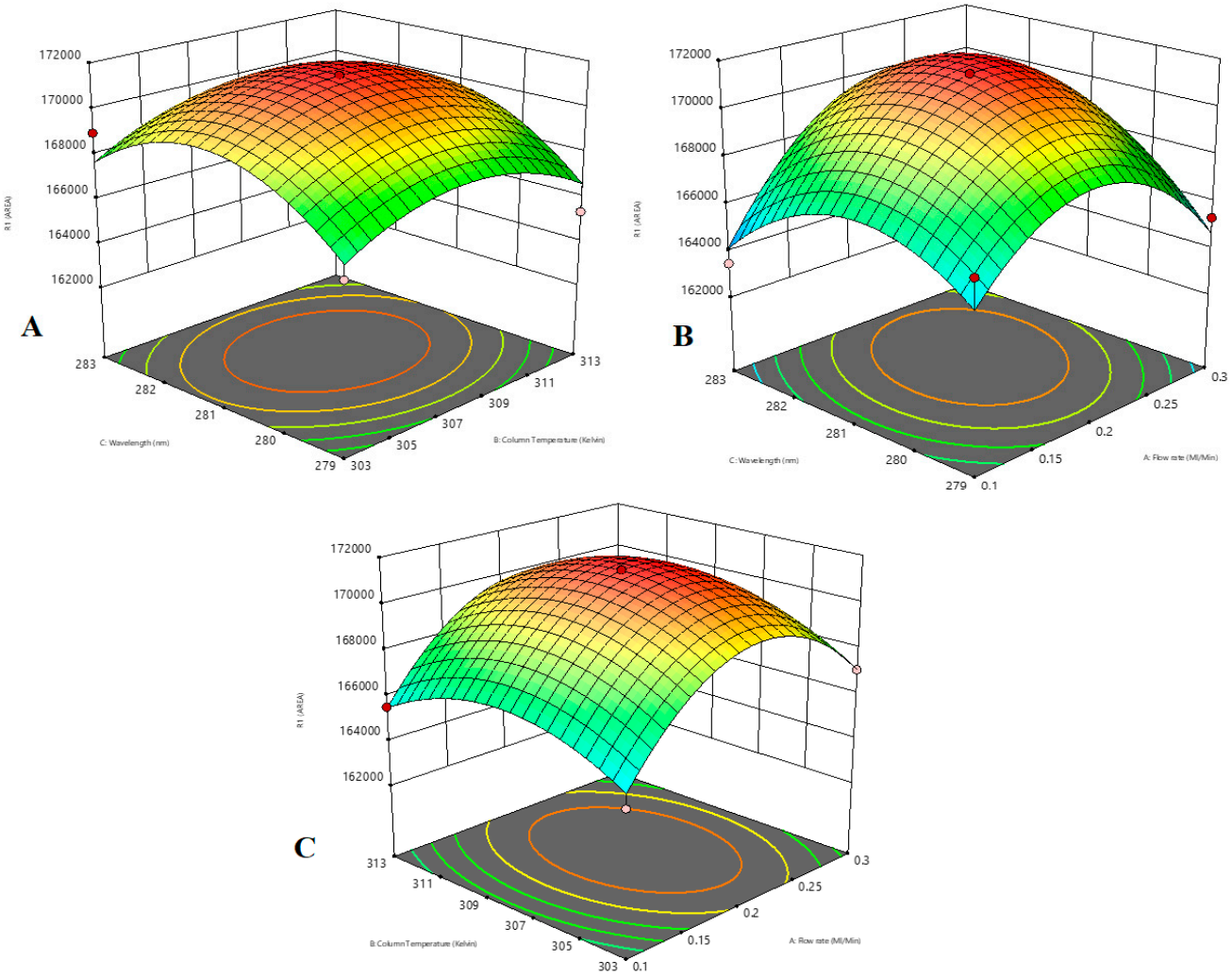

Figure 7. Three-dimensional graphs for EUG peak area. (**A**) Wavelength versus column temperature, (**B**) wavelength versus flow rate, and (**C**) column temperature versus flow rate.

The amount of EUG available in the ultrasound-assisted methanolic extracts of SA, CT, and MF was determined using the proposed UPLC technique by employing the calibration curve. The analysis of the ultrasound-assisted methanolic extracts of SA, CT, and MF using the UPLC technique showed levels of EUG of 313.67 ± 0.87 mg g^{-1}, 44.95 ± 0.56 mg g^{-1}, and 59.66 ± 0.41 mg g^{-1}, respectively.

3.2.6. Comparison with Reported Analytical Techniques

The UPLC technique was compared with several analytical procedures reported for EUG determination. The differences in the results between the proposed UPLC technique and reported analytical assays are presented in Table 5 in terms of run time, linearity range, accuracy, precision retention time, and column. For EUG assessment, the linearity, accuracy, and precision of a GC-FID method were 0.31–625 µg/mL, 88–96%, and 4.5–8.7%,

respectively [55]. These parameters were considerably lower than those of our proposed UPLC technique (linearity range, 10–100 ng/mL; accuracy, 98.93–101.51%; and precision, 0.38–1.0). Another HPLC-DAD and LC-MS/MS existing method reported a linearity range, accuracy, precision and mobile phase composition of 0.45–36 µg/mL, 99.47–101.85, 0.51–1.53 and 0.1% trifluoroacetic acid (TFA) in water and acetonitrile, respectively. All these parameters were lower than the current UPLC technique. Additionally, the composition of the mobile phase used in the above-mentioned method was buffer, but the current method used a mobile phase composition of acetonitrile:methanol:water (50:40:10, *v*/*v*/*v*) instead of buffer. Similarly, the run time, linearity range, accuracy, and precision of the existing HPLC methods for the assessment of EUG were considerably inferior to the proposed UPC technique [39,49,56]. Furthermore, existing HPLC and UPLC-DAD methods for EUG assessment exhibited poorer performance in terms of accuracy, precision and run time than the proposed UPLC-PDA technique [57]. Moreover, the linearity range of an existing HPTLC method was considerably lower than that of the established UPLC technique, but precision and accuracy results were within the acceptable range according to ICH guidelines [44,58,59]. Additionally, existing HPLC and UPLC methods for EUG assessment showed a greater retention time range (4.24–25.43 min), whereas the UPLC technique showed a retention time of EUG of 1.53 min [39,49,56,57]. However, the existing UPLC-DAD technique's linearity, accuracy, precision and run time were comparable to the current UPLC technique [57].

Table 5. Comparative evaluation of the present UPLC technique with literature analytical techniques for the estimation of EUG.

S.N.	Analytical Method	Run Time	Linearity (µg/mL)	Accuracy (% Recovery)	Precision (% RSD)	Retention Time (Min)	Column	Ref.
1	GC-FID	13	0.31–625 µg/mL	88–96	4.5–8.7	9.83	DB_{17}	[55]
2	HPLC	40	0.2–12 µg/mL	99.20	0.41–125	16.67	C_{18}	[56]
3	HPLC	60	0.45–36 µg/mL	99.47–101.85	0.51–153	25.43	C_{18}	[49]
4	HPTLC	–	532.2–8531.2 ng/band	98.25–99.32	0.34–1.09	–		[59]
5	HPTLC	–	200–1000 ng/band	99.33	1.35–1.71	–		[58]
6	HPTLC	–	100–1000 ng/band	99.3–99.8	1.71–1.85	–		[44]
7	HPLC	20	12.5–1000 ng/mL	103.7	0.27–1.19	8.43	C_{18}	[39]
8	UPLC-DAD	6	1.58–315.61 µg/mL	97–98	1.9–2.8	4.24	C_{18}	[57]
9	HPTLC	–	200–1000 ng/band	99.79	0.61–0.96	–		[60]
10	UPLC-PDA	3	10–100 ng/mL	98.93–101.51	0.38–1.0	1.53	C_{18}	CW

CW: Current work.

All of these results revealed the superiority of the proposed UPLC technique for the assessment of EUG over earlier reported HPLC, HPTLC, and UPLC techniques. Hence, the present UPLC technique can be utilized for quality control analysis in plant drug extracts, pharmaceutical preparations, and polyherbal formulations consisting of EUG as an active constituent.

3.2.7. Antioxidant Potential

The DPPH method is the most commonly used spectrophotometric technique for the estimation of the antioxidant potential of foods, beverages and extracts due to its simple, fast, and sensitive protocol. Figure 8 shows the antioxidant potential of EUG, SA, CT, MF, and standard. Ascorbic acid was used as a reference compound for antioxidant potential and the comparison of the antioxidant potential between AA and EUG as well as SA. IC_{50} values were estimated using the dose-response curve. The IC_{50} confirmed the antioxidant potential of EUG (n = 3) (IC_{50} = 3.12 µg/mL) and the methanolic extract of SA (IC_{50} = 5.97 µg/mL) in comparison to standard AA (IC_{50} = 7.06 µg/mL). The methanolic

extracts of CT (IC_{50} = 49.48 µg/mL) and MF (IC_{50} = 65.16 µg/mL) were poorer compared to ascorbic acid. Sharapov et al. reported that EUG has stronger free radical scavenging potential using DPPH (IC_{50} = 0.5 µg/mL) [61], whereas Perez et al. reported the free radical scavenging activity of eugenol by DPPH (IC_{50} = 11.7 µg/mL) [62]. On the other hand, the present study result revealed the free radical scavenging activity of EUG to have an IC_{50} = 3.12 µg/mL, which is very near to the IC_{50} = 2.98 µg/mL reported by Listler et al. [63]. This study showed the EUG and ultrasound-assisted methanolic extract of SA exhibited proton-donating abilities and could be used as free radical scavengers, which likely act as prime antioxidants.

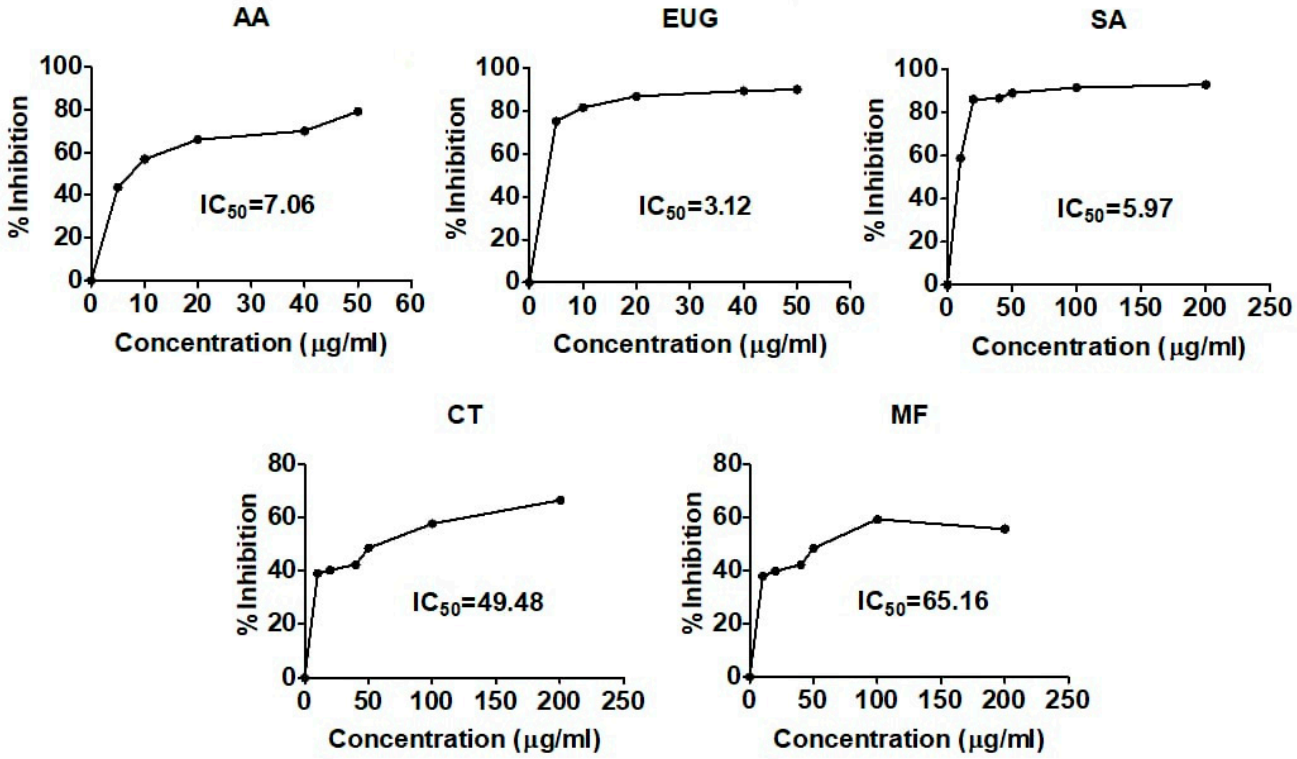

Figure 8. Scavenging effect on DPPH evident from the dose-response curve; standard ascorbic acid (AA), eugenol (EUG), *Syzygium aromaticum* (SA), *Cinnamomum tamala* (CT), and *Myristica fragrance* (MF).

4. Conclusions

This study developed and validated a rapid, precise, and robust UPLC technique for EUG assessment by using the ultrasound-assisted methanolic extracts of three spices, namely *Syzygium aromaticum*, *Cinnamomum tamala*, and *Myristica fragrance*. The UPLC technique was validated to be specific, precise, robust, and cost-effective and can be used for the detection and quantitative analysis of EUG as one of the major constituents in ultrasound-assisted methanolic extracts of crude drugs, traditional herbal formulations, and pharmaceutical preparations. The robustness of the UPLC technique was validated by minor alterations in the mobile phase flow rate, column oven temperature, and wavelength for the best-optimized condition to obtain the highest amount of EUG in ultrasound-assisted methanolic extracts of SA, CT and MF using the Box–Behenken response surface design. It is evident from the current study that the developed and validated UPLC technique can be applied for quality control analysis of a variety of plant materials, pharmaceutical products, and herbal formulations, especially Unani and Ayurvedic polyherbal formulations that have EUG as one of their major constituents. The antioxidant study results revealed that EUG and SA have strong free radical scavenging activity compared to AA, as shown in Figure 8. This study also provided a comparison of the antioxidant potential between

AA and EUG as well as the ultrasound-assisted methanolic extracts of SA, CT and MF. The current study has also demonstrated the antioxidant activity of EUG and Syzygium aromaticum, which further suggests that anticancer activity could be present due to the antioxidant activity.

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